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PIGMENTATION IN THE ORANGE TUNICATE, *ECTEINASCIDIA TURBINATA* [1]

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Ecteinascidia turbinata, a colonial ascidian of shallow waters, is characterized by the bright orange pigment in the adults' mantle tissue. The pigment is localized in fusiform organelles, approximately 3 to 6 μ in length, that are organized into stellate shaped masses heavily concentrated at the siphon end and tapering in density toward the base of the zooid, which is almost colorless. Mature oocytes and early embryos of the species are dark yellow to orange, but their pigment is diffuse, not granular, and is solubilized in yolk platelets.

The chemical nature of the pigment in *E. turbinata* has not been determined with certainty. The adult pigment was considered to be carotenoid on the basis of its similarity to the orange pigment in Synoicidae and Botryllidae species (George, 1939). The orange pigment in *Ascidia mentula* (Webb, 1939) and *Phallusia mammilliata* (Endean, 1960), however, has chemical properties entirely different from carotenoids, casting some doubt on the original interpretation based solely on comparative coloration between different species. The pigment in eggs and embryos has likewise not been subjected to direct analysis but designated "lipochrome" on the basis of its solubility in alcoholic solvents (Simkins, 1924).

Also, there has not been agreement as to whether the stellate shaped masses of the mantle pigment are true cells. Nuclei could not be found in these aggregates in hematoxylin stained paraffin sections of mantle tissue, and it was concluded that they were not cells (Simkins, 1924). George (1938), however, found methyl green stained nuclei in orange blood cells of this species and postulated that these lodged in the mantle and became permanent fixtures of this tissue.

The following studies attempt to resolve some of these questions concerning the chemical and cellular nature of the pigment in *E. turbinata.* It was felt that a better understanding of these properties of the pigment might allow some insights as to its functional role and perhaps provide a basis for any subsequent work on this little known pigmentary system.

Materials and Methods

Colonies of zooids were collected from pilings at Longbird Bridge on St. George's Island, Bermuda. They were kept in constantly flowing sea water aquaria at environmental temperatures at the Bermuda Biological Station and used for these studies within one week of their capture.

For small scale, qualitative chemical determinations, individual zooids were stripped of their tests and cut transversely into siphon and basal pieces. The

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basal ends contained stomach, hepatopancreas, heart, ovotestis, and any developing embryos within the brood pouch, while siphon ends were comprised primarily of mantle and pharyngeal tissue, with dense concentrations of mantle pigment. From 3 to 5 siphon ends were ground in a glass homogenizer with 1 ml of various solvents (see results) and centrifuged at approximately $1000 \times g$ for 10 minutes. Yellow supernatant, indicating soluble pigment, was filtered under suction using 0.45 μ pore size Millipore filters. Pigment solutions were chromatographed on 2×8 cm cellulose thin-layer sheets (Eastman Chemicals) in various developing solvents. Pigment from whole eggs and embryos was extracted, centrifuged and filtered as described, and stored at $-4°$ C in the dark. Chromatographs of these extracts were made on 2×8 cm silica-gel sheets (Eastman Chemicals) predried at 100° C for 30 minutes. The absorption spectra for egg and embryo pigment were determined with a Beckman DU spectrophotometer.

For large scale batch preparations of mantle pigment, the individual zooids were removed from the colonies and cut into siphon and basal halves. From 400 to 500 siphon halves were constantly stirred for 1 hour at room temperature in 500 ml distilled water. This separated the unpigmented tests from heavily pigmented mantle tissue, which was then rinsed of adhering water with acetone, weighed, and homogenized in 15 to 20 ml of 10% trichloracetic acid in 40% acetone using a motor driven teflon pestle with a glass homogenizer. Homogenates were centrifuged at $1000 \times g$ for 15 minutes, and the yellow supernatant filtered with the 0.45 μ Millipore apparatus.

The pigment from these extracts was purified further by first shaking 20 ml of extract over 100 ml chloroform for 24 hours at room temperature, allowing the system to stand for 12 hours at 9° C, and withdrawing the chloroform hypophase which contained no visible pigment. Any remaining acetone in the aqueous epiphase was removed by bubbling with N_2 for 10 minutes. The pigment solution was then washed 4 times with ethyl ether to remove the trichloroacetic acid, and N_2 again bubbled through it until the ether had evaporated. The resulting aqueous extract (about 12 ml) containing some precipitate and at approximately pH 6.0 was dialyzed against 1000 ml of distilled water for at least 24 hours at room temperature and with a minimum of 2 changes of distilled water. This resulted in the accumulation of a flocculent brown precipitate and the loss of yellow color in the dialysis bag. The precipitate was collected by centrifugation, dried over P_2O_5 and redissolved in 50% methanol. The yellow solution was filtered and its absorption spectrum determined on a Cary 15 recording spectrophotometer.

For ultrastructural studies, individual zooids were dissected mechanically and siphon pieces cut into quarters measuring approximately 1×3 cm. These were placed immediately into 50 ml of 6% glutaraldehyde and 3% sucrose in 0.1 M phosphate buffer at pH 7.4. They were fixed at laboratory temperatures for 4 to 6 hours, rinsed in 10 ml of cold phosphate buffer for 24 to 48 hours, and postfixed in OsO_4. Uranyl acetate and lead monoxide were used as counter-stains and the sections examined on a JEM-T6S electron microscope.

Results

Chemical analyses

Mantle pigment was not soluble in hydrophobic solvents, such as petroleum ether or hexane, or acetone, but was soluble in organic-aqueous systems includ-

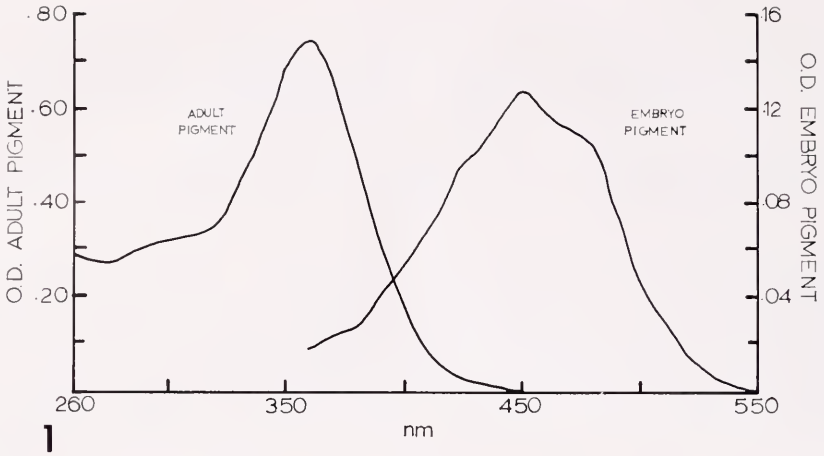

FIGURE 1. Absorption spectra of purified mantle pigment in 50% methanol (left) and embryo pigment (right) in petroleum ether. O. D. scales have been adjusted to provide curves of similar heights.

ing 70% dioxane, 50% methanol acidified to pH 3 with 1N HCl, a mixture of 4 parts butanol: 1 part acetic acid: 1 part water and 10% trichloroacetic acid in 40% acetone (TCA-acetone). Extracts in these solvents provided clear yellow solutions after filtration that remained stable at room temperatures under normal lighting conditions. The TCA-acetone extracted pigment purified as described above and redissolved in 50% methanol migrated as a single fraction of R_f 0.52 on cellulose thin-layer chromatograms using TCA-acetone as the developing solvent. The chromatograms showed no fluorescence, but the yellow pigment absorbed either long or short wave UV irradiation.

The addition of potassium borohydride to the TCA-acetone extracts to a concentration of 1 M decolorized the solution, whereas the addition of 1 M sodium nitrite did not. The TCA-acetone extracted pigment could be dialyzed using TCA-acetone as the dialyzing medium but was retained as a flocculent brown precipitate when dialyzed against distilled water.

The absorption spectrum of dialyzed pigment dissolved in 50% methanol is shown in Figure 1. The pigment absorbs strongly in the UV region, exhibiting a sharp peak at 360 nm. Absorbence in the visible range is general, shows no peaks, and falls to zero at wavelengths greater than 450 nm.

Egg and embryo pigment was soluble in acetone, petroleum ether or hexane. The extracted pigment separated into three visible fractions on silica-gel TLC using a 1 hexane: 3 ethyl acetate mixture as developing solvent. The absorption spectrum in petroleum ether for embryo pigment is shown in Figure 1. There is a major peak of 450 nm, with minor shoulders at 430 and 480 nm. The solubility of egg and embryo pigment in organic solvents, its fractionation on silica-gel chromatograms and absorbence are characteristic of carotenoids (Fox, 1953; Needham, 1974). When petroleum ether extracts of the embryo pigment were shaken with 90% methanol, the pigment remained in the ether epiphase, indicating the

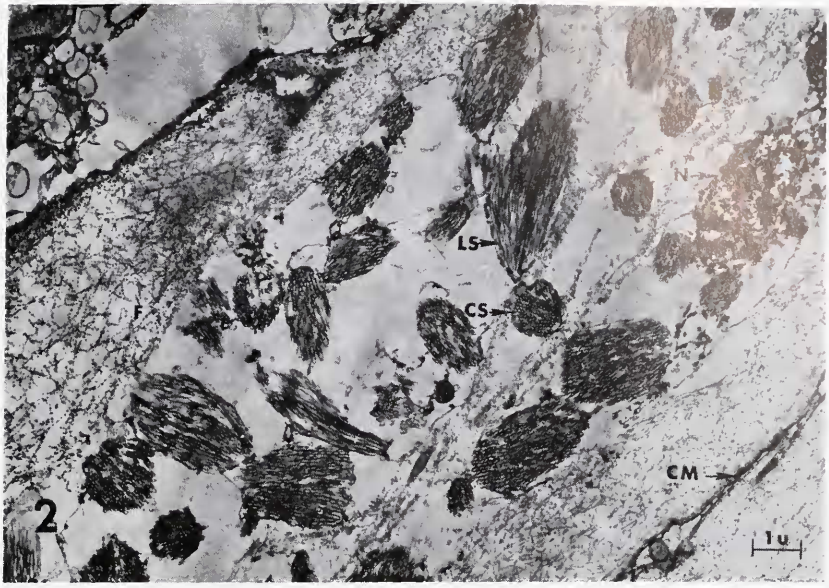

FIGURE 2. Section of pigment body mass in mantle tissue at siphon end. Pigment bodies are seen in association with an apparent nucleus (N) and may be bound by a cytoplasmic membrane (CM). The region surrounding the pigment bodies is composed of fibrils (F). Cross sections (CS) and longitudinal sections (LS) of pigment bodies are found within the same pigment body mass; 8,400 × magnification.

presence of carotenes or xanthophyll esters and the absence of xanthophylls (Fox and Vevers, 1960).

Morphological analyses

Ultrastructural characteristics of pigment bearing elements at the siphon end of the zooid are shown in Figure 2. The fusiform bodies seen at the light microscopic level appear as a structured and orderly array of tubes, approximately 500 to 600 Å in diameter, running parallel to the longitudinal axis of the organelle. It could not be determined from these studies if the tubular elements were continuous and unbranched, but cross sections of tubes from a single body were uniform in diameter (Fig. 2, CS), and this seems a likely possibility. Individual pigment organelles apparently are not bound by a limiting membrane, but this is a questionable point as no attempts were made to vary fixation and embedding procedures, and thus there is no way of determining whether those employed in this study were optimal for the demonstration of perhaps fragile membrane material.

The pigment bodies are discrete elements found within areas that may be cells or remnants of cells. The region surrounding clumps of pigment bodies is composed of a meshwork of fibrils bound by an apparent cytoplasmic membrane. The bodies are usually located in fibril free channels and occasionally associated with an apparent nucleus (Fig. 2, N).

Discussion

The orange pigment in *E. turbinata* is intense and a striking characteristic of this species. Both embryos and adults have similar coloration but differ in the morphological appearance of the pigment—diffuse in the embryo and granular in adults. The granular pigment is also found within the tissues of the ovotestis, where maturing oocytes contain their complement of diffuse pigment, giving this organ a bright orange color.

The presence of granular pigment in follicle cells surrounding development oocytes and the diffuse pigment in the larger prospective ova led Simkins (1924) to postulate that eggs became pigmented by a transfer and dissolution of granular follicular pigment into developing oocytes and that mantle pigment was derived from dissolved pigment in the yolk platelets by simply a condensation into granules during development. No chemical transformations were envisioned during these exchanges, merely the mechanical transferring of pigment to and from the yolk platelets.

The present studies have determined, however, that egg and embryo pigment is chemically dissimliar from that found in the mantle of adults. The solubilized pigment in yolk platelets is carotenoid. It is soluble in hydrophobic solvents, including petroleum ether, and has characteristic absorption properties for these compounds (Fox, 1953; Needham, 1974). On the other hand, while the precise chemical nature of the mantle pigment could not be determined, it lacks solubility and the absorbence characteristics of carotenoids and is, in general, a more stable material. Extracts of adult pigment remain unchanged even after months of storage at room temperature, are not decolorized with a strong oxidant ($NaNO_2$), and are resistant to boiling and gross changes in pH.

None of the chemical properties of the mantle pigment allow it to be placed among the better known classes of zoochromes (Needham, 1974). Its solubility in organic-aqueous systems, sensitivity to reducing agents (KBH_4) and dialysis against TCA-acetone would suggest that it is a small, polar molecule. It is not fluorescent and shows no Soret band of absorbence and, hence, does not appear to be porphyrin or pterin (Needham, 1974). Also, it does not share solubility or absorbence properties of bilichromes (Fox, 1953; 1972) or ommochromes (Butenandt, Schiedt, Bierket and Kormann, 1954).

Several attempts have been made to establish the chemical nature of the orange pigment found in blood cells of certain ascidian species which may be related to the pigment in *E. turbinata*. For example, Webb (1939) found that pigment in the orange blood cells in *Ascidia mentula* was sensitive to strong alkaline conditions but could be extracted in mineral acids. The pigment was soluble in water and acetone and resistant to oxidizing agents. Endean (1960) has also analyzed the yellow to orange pigment in blood cells of *Phallusia* (or *Ascidia;* see Webb, 1939) *mammillata*. It was resistant to strong acids, alcohol, and acetone but sensitive to 1 N NaOH. A mixture of $KMnO_4$ and H_2SO_4 also decolorized the pigment bodies, and they gave a positive argentaffin reaction. It was concluded from these observations that the pigment was a melanin.

The orange pigment in blood cells of these two species, then, has some common chemical features. It is resistant to most solvents, but sensitive to alkaline treatment. The yellow mantle pigment in *E. turbinata* is also stable and not soluble

in common organic solvents. However, it is not decolorized in 1 N NaOH and, thus, does not share this property with the pigment in the *Ascidia* species. The investigations with these species, however, were largely histochemical and made by observing the pigmented blood cells as the reagents being tested were applied directly to them. The small amount of material in the blood cells precluded extracting the pigment for more thorough, large scale, testing. It may be possible, then, that the orange pigment in the blood cells of the *Ascidia* species and in the mantle of *E. turbinata* are similar or related compounds but react differently to alkaline conditions. If the orange pigment in blood cells of the *Ascidia* species is indeed melanin (Endean, 1960), then it would appear that the mantle pigment in *E. turbinata* is not related to it. It does not have the absorbence characteristics of eumelanin (Needham, 1974) and does not change from yellow to pink in alkaline *vs.* acid conditions, as is the case for erythromelanin (Fox and Vevers, 1960). Further chemical analyses will be needed to determine the constituent elements and structure of this unusual animal pigment.

Some physical characteristics of pigment bearing corpuscles in blood cells of *A. mentula* and *A. mammillata* have also been examined. They exhibit strong birefringence, which is not completely extinguished under any conditions (Webb, 1939; Endean, 1960). Ultrastructurally the pigment bodies in *A. mammillata* are composed of flat, intercalated disks oriented lengthwise along the long axis of the organelle (Endean, 1960). In both the *Ascidia* species, then, the orange pigment bodies are elliptical structures apparently comprised of regularly arranged paracrystalline subunits.

The bodies of *E. turbinata* were also found to have a regular subunit structure, comprised of tubes running parallel to the long axis of the granule. Woollacott (1974) discovered that the pigment vesicles in the eye spot of *Ciona intestinalis* have tubular subunits, but in this case the tubes have no particular orientation and are entwined and enmeshed into compact vesicles. The pigment vesicles in this species are obviously localized in cells.

The individual stellate masses of pigment bodies in *E. turbinata* may originate from single cells or perhaps groups of cells. Bodies resembling a nucleus in size and ultrastructure were found in association with clumps of pigment bodies (Fig. 2). The pigment bodies, however, were in regions obviously larger than other cells and filled with a matrix of fibrils, apparently enclosed in a cytoplasmic membrane. Common cytoplasmic organelles such as mitochondria, endoplasmic reticulum, or lipid bodies were not seen within the fibrillar matrix. It is possible that a stellate mass of pigment bodies is derived from a cell or group of cells that becomes fixed in the mantle tissue, synthesizes pigment granules that flow into extensive cytoplasmic arms and then degenerates, leaving a nuclear remnant and cytoskeleton of fibrils filled with metabolically inactive pigment bodies. This is purely speculative but does offer some explanation for the difficulties encountered in attempting to demonstrate nuclei in these masses using standard light microscopic techniques (Simkins, 1924).

The function of orange pigment in ascidians is not known (Endean, 1960). It has been speculated that it represented an oxidative state of vanadium and that vanadium was acting as a respiratory enzyme (George, 1926; Baldwin, 1949). These hypotheses have been discounted, however, for the lack of evidence both

that the orange pigment contains vanadium and that the presence of vanadocytes enhances oxidative reactions (Webb, 1939). The orange to red pigment in the eye spot of *C. intestinalis* has not been characterized chemically, but the action spectrum for sperm release in this species is indicative of a specific photoreceptor, and the pigment has been implicated in this mechanism (Lambert and Brandt, 1967). Woollacott (1974), however, did not find any connections between the sperm duct and pigment cells in an electron microscopical study of this region and postulated instead that the pigment was acting as a barrier against the wavelengths that triggered sperm release.

The location and distribution of the orange mantle pigment in *E. turbinata* would seem to argue against its possible roles as a photoreceptor or as a respiratory catalyst. Its strong absorbence in the UV region of the spectrum may offer some protection against radiation damage to the exposed tissues at the siphon end. However, any functional role ascribed to the pigment should consider the subjectively similar color of the embryos and adults versus the chemical dissimilarities of their pigments.

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Summary

Some chemical properties of the orange pigment from eggs and embryos and the mantle tissue of adult *Ecteinascidia turbinata*, as well as ultrastructural characteristics of pigment masses in the mantle, have been examined. The diffuse egg and embryo pigment has solubility and absorbence characteristics of carotenoids, which are solubilized within yolk platelets. The granular mantle pigment, however, is not carotenoid. It is soluble only in organic-aqueous systems, stable to boiling and gross changes in pH, and absorbs strongly in the UV region with a single major peak at 360 nm. Mantle pigment is found in pigment bodies which ultrastructurally are composed of apparently straight and continuous tubular subunits, approximately 500 to 600 Å in diameter, and oriented parallel to the long axis of the body. The pigment bodies are not localized in obvious cells but found within large areas of fibrillar material that are bound by cytoplasmic membranes and occasionally contain an apparently degenerate nucleus. It may be that mantle pigment is derived from pigment cells of the blood which lose their typical cellular appearance as they become permanent features of the mantle tissue.

LITERATURE CITED

Baldwin, E., 1949. *An introduction to comparative biochemistry.* Cambridge Univ. Press, Cambridge, 164 pp.

Butenandt, A., U. Schiedt, E. Biekert, and P. Kornmann, 1954. Über Ommochrome, I. Mittelung: Isolierung von Xanthommatin, Rhodommatin and Ommatin C aus den Schupfserkreten von Vanessa urticae. *Justus Liebigs Annalen der Chemie,* **586**: 217–228.

ENDEAN, R., 1960. The blood-cells of the ascidian, *Phallusia mammillata*. *Quart. J. Microscop. Sci.*, **101**: 177–197.

FOX, D. L., 1953. *Animal biochromes and structural colours*. Cambridge Univ. Press, Cambridge, 379 pp.

FOX, D. L., 1972. Chromatology of animal skeletons. *Amer. Sci.*, **60**: 436–447.

FOX, H. M., AND G. VEVERS, 1960. *The nature of animal colours*. Sidgwick and Jackson, Ltd., London, 246 pp.

GEORGE, W. C., 1926. The histology of the blood of *Perophora viridis* (Ascidian). *J. Morphol.*, **41**: 311–331.

GEORGE, W. C., 1938. The histology of the blood of some Bermuda ascidians. *J. Morphol.*, **49**: 385–413.

GEORGE, W. C., 1939. A comparative study of the blood of tunicates. *Quart. J. Microscop. Sci.*, **81**: 391–427.

LAMBERT, C., AND C. BRANDT, 1967. The effect of light on the spawning of *Ciona intestinalis*. *Biol. Bull.*, **132**: 222–228.

NEEDHAM, A. E., 1974. *The significance of zoochromes*. Springer-Verlag New York, 429 pp.

SIMKINS, C. S., 1924. Origin of the pigment in Ecteinascidia. *Acta Zool.* **5**: 425–440.

WEBB, D. A., 1939. Observations on the blood of certain ascidians, with special reference to the biochemistry of vanadium. *J. Exp. Biol.*, **16**: 499–523.

WOOLLACOTT, R. M., 1974. Microfilaments and the mechanism of light-triggered sperm release in ascidians. *Develop. Biol.*, **40**: 186–195.